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(21) International Application Number: PCT/GB98/01026 (22) International Filing Date: 7 April 1998 (07.04.98) (30) Priority Data: 9707486.8 11 April 1997 (11.04.97) GB (71) Applicant (for all designated States except US): THE SECRETARY OF STATE FOR DEFENCE [GB/GB]; Defence Evaluation & Research Agency, Ively Road, Farnborough, Hampshire GU14 0LX (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): SQUIRRELL, David, John [GB/GB]; CBD, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). WHITE, Peter, John [GB/GB]; The Babraham Institute, Babraham Hall, Babraham, Cambridge CB2 4AT (GB). LOWE, Christopher, Robin [GB/GB]; University of Cambridge, Tennis Court Road, Cambridge, Cambridgeshire CB2 1QT (GB). MURRAY, James, Augustus, Henry [GB/GB]; University of Cambridge, Tennis Court Road, Cambridge, Cambridgeshire CB2 1QT (GB). (74) Agent: SKELTON, S., R.; D/IPR, Formalities Section (Procurement Executive), Poplar 2, MOD Abbey Wood #19, Bristol, BS34 8JH (GB).			(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: ENZYME ASSAYS			
(57) Abstract Enzymes and methods suitable for assaying ATP, and specific applications for such assays are described and claimed. In particular, there is described a recombinant mutant luciferase having a mutation (e.g. the amino-acid corresponding to amino acid residue number 245 in <i>Photinus pyralis</i> which is such that the K _m for ATP of the luciferase is increased e.g. five-fold with respect to that of the corresponding non-mutated enzyme such that it is of the order of 500 μm – 1mM. Also disclosed are luciferases having additional mutations conferring improved thermostability or altered wavelength of emitted light. Recombinant polynucleotides, vectors and host cells are also disclosed, as are methods of assaying the amount of ATP in a material (e.g. cells) optionally in real-time. Also disclosed are test-kits for <i>in vitro</i> assays.			

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ENZYME ASSAYSTechnical Field

The present invention relates broadly to enzymes and methods
5 suitable for assaying ATP. It further relates to specific
applications for such assays.

Background Art

Intracellular ATP concentrations can vary 10-fold or more
10 depending upon a cell's state of health or developmental stage.
It is of great value to be able to measure fluctuations in
intracellular ATP levels as a means of investigating e.g. the
effects of drugs, toxins, hormones, environmental agents or
disease on cells.

15 There is apparently at present no convenient method for
analysing the concentration of ATP *in vivo*. For instance, in
Dementieva et al (1996) Biochemistry (Moscow) Vol 61, No. 7.,
the intracellular concentration of ATP was measured in *E. coli*
by calculating the total amount of ATP present using a
20 recombinant luciferase, and dividing by an estimated total cell
volume.

Such an indirect approach can at best produce only an estimate
of the actual ATP concentration.

The measurement of ATP concentration in cells has also been
25 performed using an *in vitro* coupled assay, such as that
disclosed in the Sigma Diagnostic Kit Catalog No. 366, in which
Phosphoglycerate kinase is used to convert 3-phosphoglycerate
to 1,3 diphosphoglycerate in an [ATP]-dependent fashion. The
1,3 diphosphoglycerate is then converted to glyceraldehyde-3-P
30 concomitantly with conversion of NADH to NAD, which can be
monitored spectroscopically. The assay has a dynamic range up
to 1 mM; the expected range is 380-620 μ M when used with blood
cells.

However it can be seen that, as with all coupled assays, the test is inevitably cumbersome to perform. Additionally it could not readily be adapted for *in vivo* use. It would thus be a contribution to the art to provide materials and methods which
5 overcome some of the drawbacks of the prior art.

Disclosure of the Invention

In a first aspect of the invention there is provided a recombinant mutant luciferase having a mutation which is such
10 that the K_m for ATP of the luciferase is increased with respect to that of the corresponding non-mutated enzyme. Preferably the K_m is at least double that of the non-mutated enzyme, and more preferably at least around five, ten, or twenty times higher than that of the non-mutated enzyme.

15 Luciferases are, of course, already known in the art. In the presence of Mg^{2+} , luciferase (originally obtained from fireflies) catalyzes the reaction of luciferin, ATP and O_2 to form oxyluciferin, AMP, CO_2 , pyrophosphate and light. This basic property (luciferin and ATP to produce light) is
20 hereinafter referred to as 'luciferase activity'.

The term 'luciferase' as used in relation to the invention is intended to embrace all luciferases, or recombinant enzymes derived from luciferases which have luciferase activity. This explicitly includes recombinant mutant luciferases which have
25 deletions, additions or substitutions to their amino acid structure provided that they retain luciferase activity. Such luciferases will typically have considerable homology (e.g. up to 70, 80, 90, or 99%) with wild-type enzymes. However the crucial technical feature of the luciferases of the present
30 invention which distinguishes them from those of the prior art is that they have a mutation which causes an increase in the K_m for ATP of the luciferase as compared with that measured for a corresponding enzyme which differs only in that it lacks that same mutation.

This increase K_m may be measured by the person of ordinary skill in the art by conventional enzyme assays, as described in more detail in the Examples below.

It should be noted that in the prior art, luciferase has sometimes been used as a marker for gene expression (in vivo) where its production in a cell is linked to a particular genetic control element. Luciferin is added exogenously and intracellular ATP concentrations, under almost all conditions, will be such that the enzyme is saturated. Thus the switching on of gene expression is signalled by light that is emitted in a quantitative manner according to the amount of active luciferase that is generated.

However it should be stressed that in the previously known systems it is generally the concentration of luciferase which is measured; this concentration is then correlated with a different event e.g. the efficiency of a promoter. Indeed it has, on occasions, been an object of the prior art teaching on luciferases to reduce the K_m for ATP (see e.g. WO 96/22376) which ensures that changes in the ambient [ATP] does not interfere with the assay.

Similarly the assay disclosed by Dementieva et al (1996) discussed above requires that all of the ATP be efficiently converted to light so that the total ATP present can be calculated. This approach requires a low K_m luciferase so that the enzyme operates at near maximal velocity until all the ATP is hydrolysed.

By making available luciferases which have an increased K_m compared with those already known in the art, the present inventors have for the first time opened up the possibility of using these enzymes to measure steady state ATP concentrations over range which was previously unsuitable. This is because, generally speaking, the relationship between enzyme velocity (V , as measured by light intensity) and substrate concentration (of ATP, where luciferin is in excess) is as follows:

$$V = V_m \cdot [ATP] / K_m + [ATP]$$

It can therefore be seen that only when the K_m is greater than (or of a similar order as) the ambient [ATP] will there be a degree of proportionality between changes in [ATP] and changes in light intensity. Where the K_m is much less than the ambient [ATP], any changes in [ATP] will not tangibly effect the measured light intensity. Clearly the more sensitive the light detection is, the smaller the measurable changes in 'V' can be, and the smaller the K_m can be with respect to the [ATP] range being assessed.

For certain applications, e.g. *in vivo* measurements, it may be advantageous to have a luciferase wherein the K_m is of the order of between 400 μ M to 1.4 mM e.g. 500 μ M, 600 μ M, 1 mM etc. However, as can be appreciated from the discussion above, the main criterion is that the K_m is not much less than the expected [ATP] range to be assessed, and the phrase 'of the order of' should be construed accordingly.

A particular expected [ATP] range which is important for physiological assays of blood cells is between 300 μ M and 1 mM, or more particularly 380 μ M and 620 μ M, (cf. Sigma Diagnostic Kit, Catalog No. 366 discussed above). For other mammalian cells such as hepatocytes, the [ATP] range is 2.5 mM - 6 mM (see Dementieva et al (1996) discussed above. Use of the recombinant luciferases of the present invention for continuous assays in these ranges is particularly envisaged.

The disclosure of the present application makes such high K_m luciferases available for the first time. The prior art disclosures reveal only luciferases having a K_m of between 60 μ M and 150 μ M, which would be saturated in these ranges.

It is also advantageous, as with all enzymes used in assays, that the mutant enzyme retains sufficient activity (i.e. a high maximum turnover number, giving a high V_m) such that practical concentrations of enzyme can give detectable results.

Preferably the activity for ATP of the mutant is at least 5-100% of that of the corresponding wild-type; however reduced-activity as a result of the high K_m mutation can, if necessary, be compensated for by using more enzyme or more sensitive
5 detection if required.

In one embodiment of the first aspect there is disclosed a luciferase wherein the amino-acid corresponding to amino acid residue number 245 in *Photinus pyralis* has been substituted with respect to the corresponding wild-type amino acid residue
10 such that the K_m for ATP is increased with respect to that of the corresponding non-mutated enzyme.

It should be noted that the sequences of a number of luciferases from different sources have already been published in the literature, see e.g. WO 95/25798 for *P pyralis*; EP 0 524
15 448 for *Luciola cruciata* and *Luciola lateralis*. Other known luc genes include *Luciola mingrelica*, and *Lampyrus noctiluca* (see Newby et al (1996) Biochemical J 313: 761-767.)

Whether an amino-acid in a luciferase 'corresponds' to number 245 in *P pyralis* (which is His in the wild-type, non-mutated
20 enzyme) can be established by the person of ordinary skill in the art without difficulty as follows: the sequence of the luciferase is established (either from the literature or by sequencing); the sequence is aligned with *P pyralis*, for instance using commercially available software (e.g. "Bestfit"
25 from the University of Wisconsin Genetics Computer Group; see Devereux et al (1984) Nucleic Acid Research 12: 387-395) or manually such as to demonstrate maximal homology and align conserved amino acids; the amino acid corresponding to number 245 in *P pyralis* is identified. An example of this is shown
30 below using *L cruciata* - the corresponding amino acid in that case is number 247.

Once identified a mutant can be prepared e.g. by site directed mutagenesis by methods commonly used in the art and exemplified below.

Preferably corresponding amino-acid is substituted for an uncharged amino acid, for instance nonpolar (e.g. Ala) or uncharged polar (e.g. Asn, or Gln):

<u>CLASS</u>	<u>EXAMPLES OF AMINO ACID</u>
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5	Nonpolar:	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
	Uncharged polar:	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
	Acidic:	Asp, Glu
	Basic:	Lys, Arg, His

It should be noted that WO 95/18853 (PROMEGA) lists a large
10 number (over 80) of *Pyrophorus plagiophtalamus* mutants which are reported to have altered spectral properties. However the K_m for ATP of the mutants is not reported, nor indeed discussed at any point in the application.

In another embodiment of the first aspect there is disclosed a
15 luciferase wherein the amino-acid corresponding to amino acid residue number 318 in *Photinus pyralis* has been substituted with respect to the corresponding wild-type amino acid residue such that the K_m for ATP is increased with respect to that of the corresponding non-mutated enzyme. Correspondence may be
20 assessed as above; preferably the amino acid (Ser in the wild type) is substituted for a bulkier one (e.g. Thr).

In preferred forms the mutant luciferases of the present invention incorporate one or more further mutations capable of conferring one or more of the following properties with respect
25 to a corresponding non-mutated enzyme: improved thermostability; altered wavelength of emitted light. Some suitable mutations are already known to those skilled in the art; see e.g. WO 95/25798 and WO 96/22376 and EP 0 524 448 for thermostability improving mutations (e.g. at positions
30 corresponding to 354 and 215 of *P pyralis*).

Preferably the mutation causing the increased K_m itself improves one or more of these properties, particularly thermostability. It should be noted that an enhanced stability at around 37°C is especially advantageous for enzymes which are to be employed in vivo.

In a further embodiment the luciferases may be in the form of fusion proteins or incorporate polypeptide extensions. This may improve the ease by which they can be produced, localised in vivo or extracted and purified.

- 10 In a second aspect of the invention there is disclosed a recombinant polynucleotide encoding a mutant luciferase of the present invention, as described above.

- In a third aspect there are disclosed vectors comprising a polynucleotide of the second aspect. For instance vectors
15 further comprising a replication element which permits replication of the vector in a suitable host cell and/or a promoter element which permits expression of said polynucleotide in a suitable host cell. The promoter may be a constitutive promoter. Optionally the promoter element may be
20 tissue- or organ-specific.

In a fourth aspect there is disclosed a host cell containing, or transformed with, a vector of the third aspect.

- Optionally the host cell of the fourth aspect may express one or more further luciferases which have a lower K_m for ATP than
25 those of the present invention, and possibly emit light of a different wavelength, such as to extend the useful range of any assay, and/or allow the use of a ratiometric assay i.e. one in which the activity of the high K_m mutant is compared with that of a further luciferase. The further luciferases may be
30 recombinant non-mutant luciferases or recombinant mutant luciferases having a mutation which is such that the K_m for ATP of the luciferase is decreased with respect to that of the corresponding non-mutated enzyme (see e.g. WO 96/22376 .

Coloured mutants are disclosed in WO 95/18853 and in Ohmiya et al (1996) FEBS Letters 384: 83-86.

In a fifth aspect there is disclosed a process for producing a luciferase of the present invention comprising culturing a host
5 cell as described in the fourth aspect.

In a sixth aspect there is disclosed a single cell organism consisting of a host cell as described above, or a multicellular organ or organism comprising it. The use of e.g. transgenic higher animals in which the luciferases of the
10 present invention are expressed could allow *in vivo* study of [ATP] in different types of cell or tissue as described in more detail below. In particular, as ATP is present in virtually all living cells, any type of cell into which luciferase could be cloned, from bacterial to plant or animal, could be studied
15 through the measurement of ATP changes.

Thus in a seventh aspect of invention there is disclosed a method of assaying the amount of ATP in a material comprising use of a recombinant luciferase as described above.

Preferably the method comprises the following steps (a) the
20 luciferase is contacted with the material and luciferin; (b) the intensity of light emitted by the luciferase is measured; and (c) the measurement in step (b) is correlated with the amount of ATP in the material.

The measurement in step (b) may be compared with a control
25 value such as minimise base-line errors.

The assay can be *in vitro* or *in vivo*.

More preferably the material itself is a cell, in to which the luciferase is introduced e.g. by transforming the cell with a vector as described above. Alternatively the luciferase may be
30 introduced into the cell by direct injection.

Equally the material measured may be part of a synapse i.e. the ATP is neurotransmitter.

Generally the assay will be most useful for real-time analysis (on a time-scale of seconds e.g. using a CCD camera, photomultiplier or photodiode) of events initiated by particular stimuli (e.g. addition of an active agent to the material). In this case the assay can monitor changes in [ATP] concentration over a relatively short time-scale. Such measurement will not, therefore, be greatly affected by longer time-scale events, such as changes in the concentration of luciferase in the system. These changes can be correlated with cellular events e.g. tissue necrosis may be associated with falling [ATP], fatigue in muscle likewise. Such continuous assays have hitherto not been possible.

Other possible applications include measuring the effect of drug treatments on various tissues; toxins and uncoupling agents on oxidative phosphorylation; bacterial infection; metabolic processes and stress (e.g. obesity and exercise); studies of brain activity (e.g. memory function and mental disorders) etc.

If appropriate the [ATP] can be measured periodically (rather than constantly) using photographic film.

Essentially the monitoring can be done in ways analogous to those already used in the art for other applications e.g. for the photonic detection of bacterial pathogens in living hosts disclosed by Contag et al (1995) *Molecular Microbiology* 18(4): 593-603. In that paper a Hamamatsu intensified CCD camera was used to visualise *Salmonella Typhimurium* expressing luciferase during infection of a mouse. Equally a system equivalent to PET (positron emission tomography - as used in brain scans) could be used to achieve precise localisation of luciferase-generated light to allow the metabolism of specific body regions to be ascertained.

Generally speaking it will be necessary to introduce luciferin into the system being studied. By 'luciferin' is meant any co-factor which has luciferin activity i.e. can be used in conjunction with luciferase to cause light to be emitted in the presence of ATP. The manner by which this is introduced in to the system will depend on the system itself. For instance where animal cells are being studied, luciferin may be introduced by ingestion of luciferin or a precursor thereof by an animal of which the cell is a constituent part. Similarly when the system being studied is one or more plant cells, the luciferin may simply be introduced into the cell by applying a solution of luciferin or a precursor thereof to a plant of which the cell is a constituent part.

In a final aspect of the invention there is disclosed a test kit comprising a luciferase discussed above and further comprising one or more of the following (a) a buffer or dry materials for preparing a buffer; (b) ATP standards; (c) luciferin; (d) Dithiothreitol (e) instructions for carrying out an ATP assay.

The invention will now be further described with reference to the following none-limiting Figures, Sequence Listings and Examples.

Figures

Fig 1. shows plasmid pPW601a as described in Example 1.

Fig 2. for mutant H245A, (a) shows the plot of V against [ATP] and (b) shows 1/V against 1/[ATP] as described in Example 1.

Fig 3. shows a sequence comparison of one region of *P pyralis* (Pp) and the corresponding region of *L cruciata* (Lc) as described in Example 2.

Fig 4 is a graph showing light emission versus ATP concentration for mutant H245N.

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Fig 5 . shows the effect of the addition of nutrient broth to luciferase-expressing *E. coli* cells pre-charged with Luciferin as described in Example 6.

Fig. 6 shows Seq ID No. 1 which is the nucleotide sequence of the wild type *luc* gene from *P. pyralis*.

Fig. 7 shows Seq ID Nos. 2-5 which are the primers used to create the mutations H245A, N and Q (Ala, Asn, or Gln - see Seq ID Nos. 2, 3 & 4) and the equivalent wild-type sequence (Seq ID No 5).

Fig. 8 shows Seq ID No. 6 which is the amino acid sequence of a high K_m mutant H245Q of the present invention, wherein amino acid 245 has been changed to Gln.

Examples

15

EXAMPLE 1: PRODUCTION OF RECOMBINANT HIGH K_m MUTANT LUCIFERASE

Except where otherwise stated, the methods employed were as those used by White et al (1996) Biochemical Journal 319: 342-350, which is concerned with thermostable mutants.

STARTING MATERIALS: Mutants were generated by site directed mutagenesis of the plasmid pPW601a (Fig 1) comprising the luciferase gene, *luc*, from *P. pyralis*. The wild type *luc* gene from *P. pyralis* is shown at Seq ID No. 1. Plasmid pPW601a was created by cloning the *luc* gene *Bam*HI/*Sst*I fragment from pGEM-*luc* (available from Promega) into pDR540 (available from Pharmacia). The unique *Xho*I site in the polylinker of the plasmid was removed to simplify the following procedures.

SITE DIRECTED MUTAGENESIS: Three mutagenic oligonucleotides were used to create the mutations H245A, N and Q (Ala, Asn, or Gln - see Seq ID Nos. 2, 3 & 4). The equivalent wild-type sequence is shown at Seq ID No. 5. The oligonucleotides also introduced a silent mutation which destroys a unique *Xmn*I site in the *luc* gene - this did not result in an amino acid

substitution but facilitated mutant selection. The mutagenesis was carried out in accordance with the kit instructions of kit supplied by Clontech laboratories Inc, Palo Alto, California USA.

- 5 The amino acid sequence of H245Q is shown in Seq. ID No. 6.

ISOLATION OF PLASMID DNA & TRANSFORMATION: this was carried out by the method of Brinboim & Doly (1979) Nucleic Acids Research 7: 1513.

- 10 CELL CULTURE AND EXTRACTION: *E. coli* JM109 transformants were grown to an $OD_{600} = 1.0$. Aliquots of cells expressing mutant luciferases from plasmid pPW601a, were subjected to lysis as described in the Promega technical bulletin and the lysed extracts were then stored on ice prior to assay.

- 15 ASSAY OF K_m OF MUTANT LUCIFERASES: luciferase assays were performed at 21°C using 100 μ l of assay buffer (20 mM Tricine pH 7.8 containing 2.0 mM $MgSO_4$, 0.1 mM EDTA, 33mM dithiothreitol, 470 μ M D-luciferin and ATP in the concentration range 6.25 - 800 μ M). Each assay contained 5-10 μ l of crude cell extract.

- 20 The plots of V against [ATP] and 1/V against 1/[ATP] for mutant H245A are shown in Fig. 2. Such plots can be used to determine the K_m .

The results of each mutation and the recombinant Wild Type are shown in Table 1:

Table 1

Luciferase	K _m MgATP (μ M)
r Wild Type	66
H245A	442
H245N	623
H245Q	1340
A215L*	65

* A215L is a thermostable mutant in which amino acid 215 is substituted with lysine (see WO 96/22376 - SECRETARY OF STATE FOR DEFENCE).

ASSAY OF THERMOSTABILITY OF MUTANT LUCIFERASES: the thermostability of H245N & H245Q was also tested, as compared with mutant A215L and the wild-type. Lysed crude extracts of cells containing luciferase activity were incubated at 37°C for set time periods. The thermostability of the mutant H245A was found to be very similar to that of the recombinant wild-type. The results are shown in Table 2:

Table 2

Enzyme	Remaining activity %			
	0	2	4	8 minutes
r Wild Type	100	64.8	36.6	26.6
A215L	100	101	88	84
H245N	100	96	61	46
H245Q	100	103	78.6	51.5

PURIFICATION: luciferases, e.g. incorporating the H245Q mutation, may be purified as described in White et al (1996) [supra]. Briefly, the cell lysates are centrifuged at 30000 g for 30 mins and the supernatant is fractionated with ammonium sulphate (30-55%). This fraction is resuspended and desalted. The desalted material was passed through a hydroxyapatite column and eluted with 10-200 mM sodium phosphate containing dithiothreitol. The luciferase containing eluant is dialysed and applied to a Mono Q anion-exchange column. The enzyme can be eluted with 0 to 500 mM NaCl.

EXAMPLE 2: IDENTIFICATION OF CORRESPONDING HIGH K_m MUTANTS

Fig 3. shows a sequence comparison of one region of *P pyralis* and the corresponding region of *L cruciata* as describe din Example 2. In this case it can be seen that amino acid 245 corresponds to 247.

EXAMPLE 3: EXPRESSION OF MUTANT LUCIFERASE IN MAMMALS

This can be achieved by methods analogous to those disclosed by Liu et al (1997) Nature Biotechnology 15: 167-173. In this method cationic liposomes are used to deliver plasmid DNA containing luciferase a gene to mice.

EXAMPLE 4: AN IN VIVO ATP ASSAY IN MAMMALS

This can be carried out by methods analogous to those used by Contag et al (1995) Molecular Microbiology 18(4): 593-603. In this method luciferase expression in *S typhimurium* in mice is monitored using a CCD camera.

EXAMPLE 5: A KIT FOR AN IN VITRO ATP ASSAY

This may be provided as follows: luciferase H245Q; buffer; or dry materials for preparing a buffer; ATP for standards; luciferin; and instructions for carrying out an ATP assay.

10 EXAMPLE 6 : Assay for determining cell behaviour

Using a luciferase assay as described in Example 1, a plot of the photon count versus the ATP concentration was prepared for the H245N mutant. The results are shown in Figure 4.

In order to demonstrate how the enzyme of the invention can be used in studying cellular behaviour, a sample of recombinant E.coli cells which expressed the H245N mutant luciferase were rendered dormant by exhaustion of nutrients. The cells were charged with luciferin by 10 minutes immersion in p.H. 5.0 citrate buffer containing 1mM luciferin. They were then centrifugally washed, resuspended in 1ml Nutrient Broth and the luminescence monitored. The results are shown in Figure 5.

Using the mutant luciferase of the invention, the revival and growth of functional cells could be monitored.

CLAIMS

1. A recombinant mutant luciferase having a mutation which is such that the K_m for ATP of the luciferase is increased with respect to that of the corresponding non-mutated enzyme.
2. A luciferase as claimed in claim 1 wherein the K_m is at least double that of the non-mutated enzyme.
3. A luciferase as claimed in claim 2 wherein the K_m is at least five times higher than that of the non-mutated enzyme.
4. A luciferase as claimed in claim 1 wherein the K_m is of the order of 500 μ M.
5. A luciferase as claimed in claim 1 wherein the K_m is of the order of 1 mM.
6. A luciferase as claimed in any one of the preceding claims having a V_m for ATP which is at least 5-100% of that of the corresponding wild-type.
7. A luciferase as claimed in any one of the preceding claims wherein the amino-acid corresponding to amino acid residue number 245 in *Photinus pyralis* has been substituted with respect to the corresponding wild-type amino acid residue such that the K_m for ATP is increased with respect to that of the corresponding non-mutated enzyme.
8. A luciferase as claimed in claim 7 wherein the amino-acid has been substituted for an uncharged amino acid.
9. A luciferase as claimed in claim 8 wherein the amino-acid has been substituted for Ala, Asn, or Gln.
10. A luciferase as claimed in any one of claims 7 to 9 which is derived from *Photinus pyralis* and wherein amino acid residue number 245 has been substituted.

11. A luciferase as claimed in any one of claims 7 to 9 which is derived from *Luciola cruciata* and wherein amino acid residue number 247 has been substituted.
12. A luciferase as claimed in any one of the preceding claims incorporating one or more further mutations capable of conferring one or more of the following properties with respect to a corresponding non-mutated enzyme: improved thermostability; altered wavelength of emitted light.
13. A fusion protein comprising a luciferase as claimed in any one of the preceding claims.
14. A recombinant polynucleotide encoding a luciferase as claimed in any one of the claims 1 to 12.
15. A replication vector comprising a polynucleotide as claimed in claim 14 further comprising a replication element which permits replication of the vector in a suitable host cell.
16. An expression vector comprising a polynucleotide as claimed in claim 14 further comprising a promoter element which permits expression of said polynucleotide in a suitable host cell.
17. A vector as claimed in claim 16 wherein the promoter element is tissue or organ specific.
18. A host cell containing a vector as claimed in any one of claims 15 to 17.
19. A host cell transformed with a vector as claimed in any one of claims 15 to 17.
20. A host cell as claimed in claim 19 which also expresses a second luciferase having a lower K_m for ATP.
21. A host cell as claimed in claim 20 wherein the second luciferase is selected from: (a) a recombinant non-mutant

luciferase; (b) a recombinant mutant luciferase having a mutation which is such that the K_m for ATP of the luciferase is decreased with respect to that of the corresponding non-mutated enzyme.

22. A process for producing a luciferase comprising culturing a host cell as claimed in any one of claims 19 to 21.

23. A host organism consisting of or comprising a host cell as claimed in any one of claims 19 to 21.

24. Use of a recombinant luciferase as claimed in any one of claims 1 to 12 for assaying the amount of ATP in a material, wherein the concentration of the ATP is expected to be between 300 μ M and 6 mM.

25. A method of assaying the amount of ATP in a material comprising use of a recombinant luciferase as claimed in any one of claims 1 to 12.

26. A method as claimed in claim 25 wherein (a) the luciferase is contacted with the material and luciferin; (b) the intensity of light emitted by the luciferase is measured; and (c) the measurement in step (b) is correlated with the amount of ATP in the material.

27. A method as claimed in claim 26 wherein the measurement in step (b) is compared with a control value.

28. A method as claimed in claim 26 wherein the measurement in step (b) is made in real-time.

29. A method as claimed in any one of claims 25 to 28 wherein the material measured forms part of a synapse.

30. A method as claimed in any one of claims 25 to 28 wherein the material is a cell and the luciferase is introduced into the cell.

31. A method as claimed in claim 30 wherein the luciferase is introduced into the cell by transforming the cell with a vector as claimed in any one of claims 15 to 17.

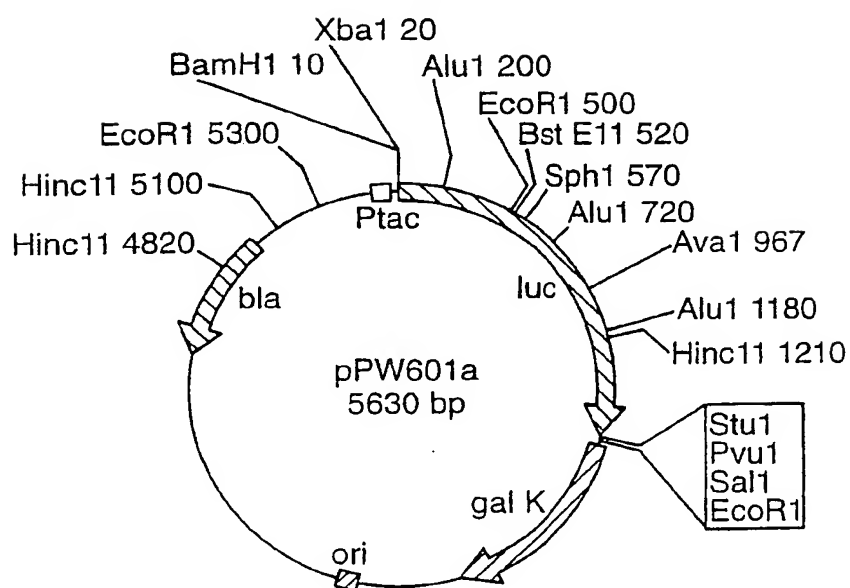
32. A method as claimed in claim 30 or claim 31 wherein the luciferin is introduced into the cell by direct injection.

33. A method as claimed in claim 30 or claim 31 wherein the cell is an animal cell and the luciferin is introduced into the cell by ingestion of luciferin or a precursor thereof by an animal of which the cell is a constituent part.

34. A method as claimed in claim 30 or claim 31 wherein the cell is a plant cell and the luciferin is introduced into the cell by applying a solution of luciferin or a precursor thereof to a plant of which the cell is a constituent part.

35. A test kit comprising a luciferase as claimed in any one of claims 1 to 12 and further comprising one or more of the following (a) a buffer or dry materials for preparing a buffer; (b) two or more measured portions of ATP suitable for preparing standard solutions; (c) luciferin; (d) instructions for carrying out an ATP assay.

Fig.1.



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Fig.2.

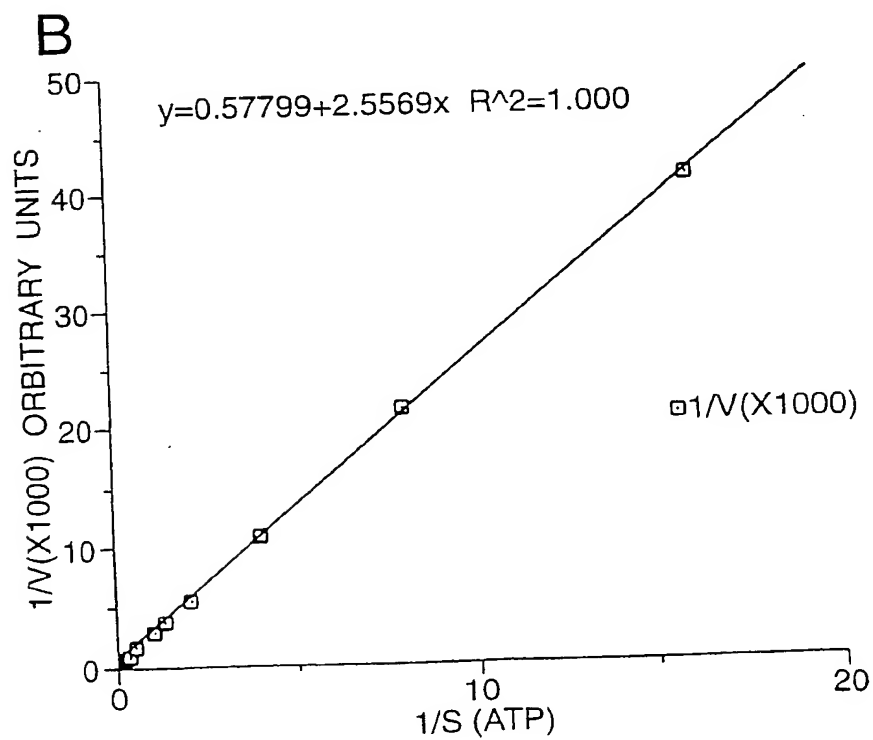
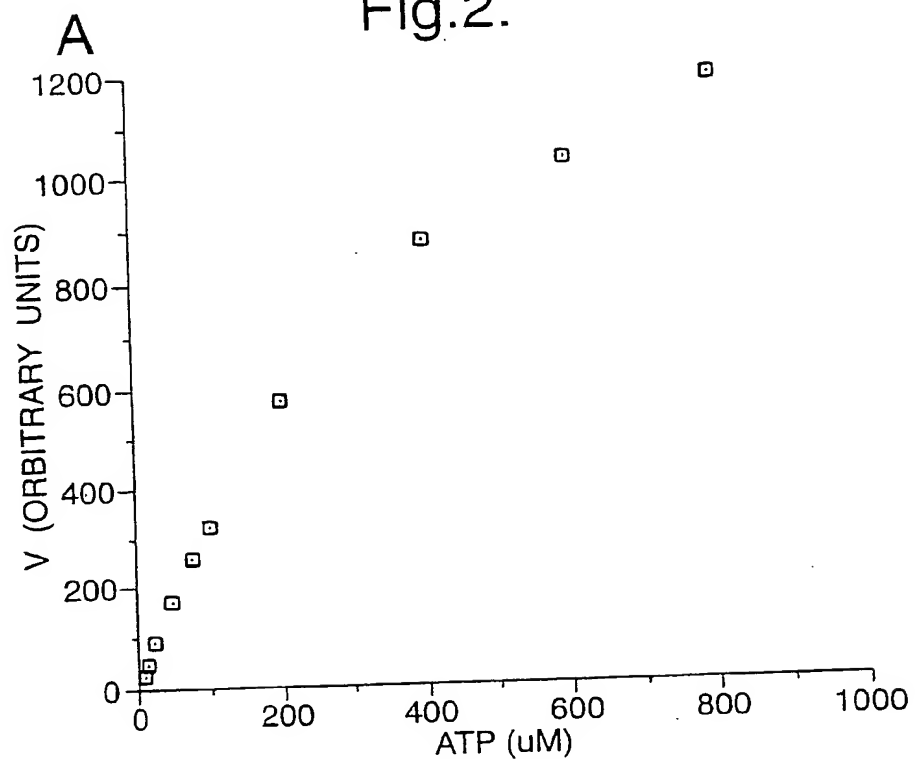


Fig. 3.

Fig. 3.

230	↓	H245	
Pp	IIPD	TAILSVVPFHHGFGMFTTLGYLIC	23/28 identical
Lc	VSPGTAVLTVVPFHHGFGMFTTLGYLIC		
232	↑	247	
301	↓	P318A	
Pp	IDKYDLSNLHEIASGGAPLSKEVGEAVAKRFHLPG		30/35 identical
Lc	LNKYDLSNLVEIASGGAPLSKEVGEAVARRFHLP		
303	↑	320	
330	↓	S347T	
Pp	RFHLPGI RQGYGLTETTSAILITPEGDDKPGAVGKVVPFF		
Lc	RFNLPGVRRQGYGLTETTSAILITPEGDDKPGASGKVVPPLF		35/40 identical
332	↑	349	

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Fig.4.

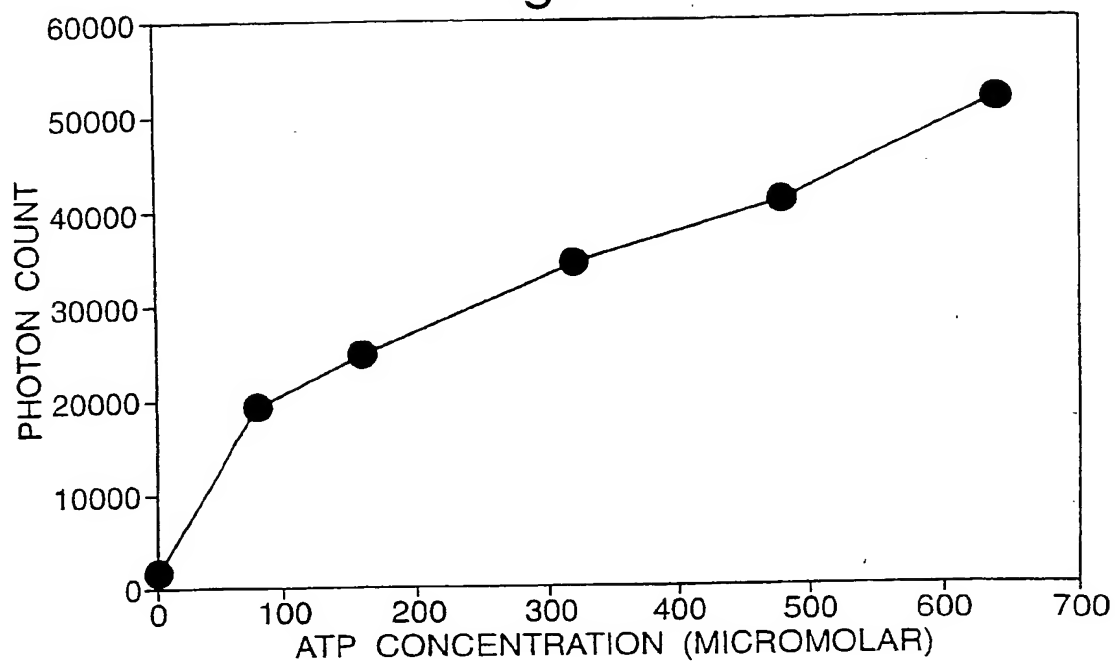
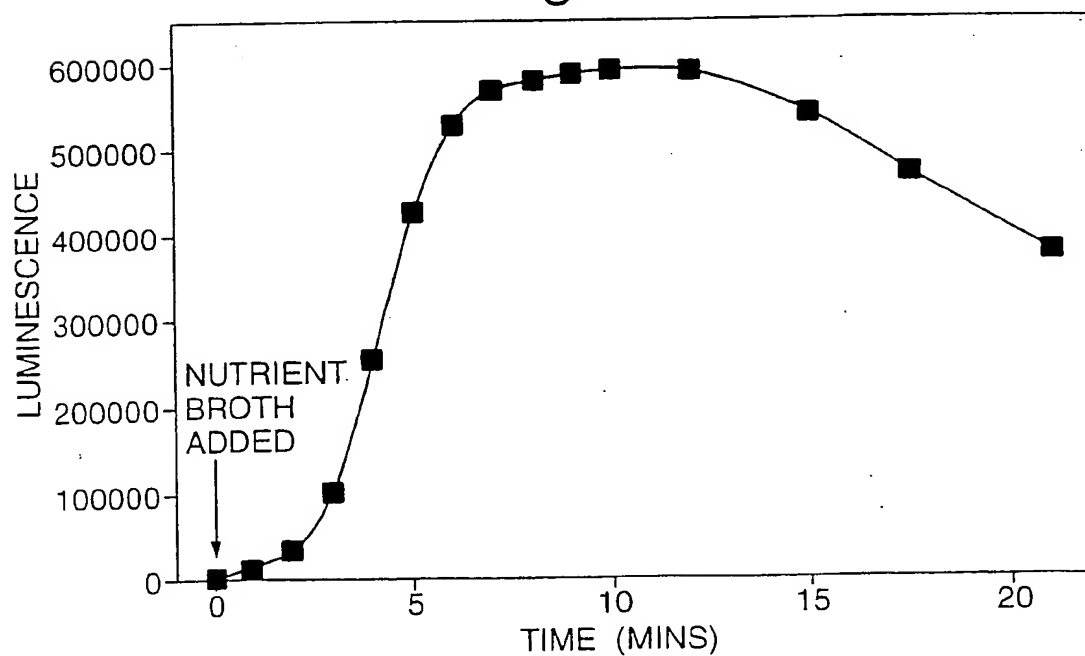


Fig.5.



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Fig.6.

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1722 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Photinus pyralis*

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 4..1653

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATTGCTTTTA CAGATGCACA TATCGAGGTG AACATCACGT ACGCGGAATA CTTCGAAATG	180
TCCGTTTCGGT TGGCAGAAGC TATGAAACGA TATGGGCTGA ATACAAATCA CAGAATCGTC	240
GTATGCAGTG AAAACTCTCT TCAATTCTTT ATGCCGGTGT TGGGCGCGTT ATTTATCGGA	300
GTTGCAGTTG CGCCCGCGAA CGACATTTAT AATGAACGTG AATTGCTCAA CAGTATGAAC	360
ATTTCCGAGC CTACCGTAGT GTTTGTTTCC AAAAAGGGGT TGCAAAAAAT TTTGAACGTG	420
CAAAAAAAT TACCAATAAT CCAGAAAATT ATTATCATGG ATTCTAAAAC GGATTACCAG	480
GGATTTTCAGT CGATGTACAC GTTCGTCACA TCTCATCTAC CTCCCGGTTT TAATGAATAC	540
GATTTTGTAC CAGAGTCCTT TGATCGTGAC AAAACAATTG CACTGATAAT GAATTCCTCT	600
GGATCTACTG GGTACCTAA GGGTGTGGCC CTTCCGCATA GAACTGCCTG CGTCAGATTC	660
TCGCATGCCA GAGATCCTAT TTTTGGCAAT CAAATCATTC CGGATACTGC GATTTTAAGT	720

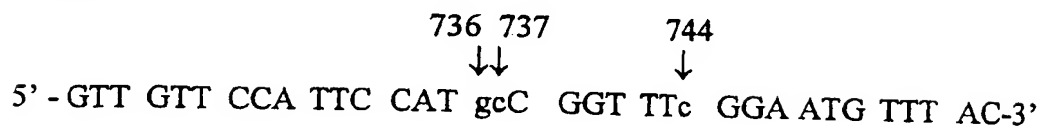
Fig.6 (Cont).

GTTGTTCCAT TCCATCACGG TTTTGAATG TTTACTACAC TCGGATATTT GATATGTGGA	780
TTTCGAGTCG TCTTAATGTA TAGATTTGAA GAAGAGCTGT TTTTACGATC CCTTCAGGAT	840
TACAAAATTC AAAGTGC GTT GCTAGTACCA ACCCTATTTT CATTCCTTCGC CAAAAGCACT	900
CTGATTGACA AATACGATTT ATCTAATTTA CACGAAATTG CTTCCTGGGGG CGCACCTCTT	960
TCGAAAGAAG TCGGGGAAGC GGTGCAAAA CGCTTCCATC TTCCAGGGAT ACGACAAGGA	1020
TATGGGCTCA CTGAGACTAC ATCAGCTATT CTGATTACAC CCGAGGGGGA TGATAAACCG	1080
GGCGCGGTCTG GTAAAGTTGT TCCATTTTTT GAAGCGAAGG TTGTGGATCT GGATACCGGG	1140
AAAACGCTGG GCGTTAATCA GAGAGGCGAA TTATGTGTCA GAGGACCTAT GATTATGTCC	1200
GGTTATGTAA ACAATCCGGA AGCGACCAAC GCCTTGATTG ACAAGGATGG ATGGCTACAT	1260
TCTGGAGACA TAGCTTACTG GGACGAAGAC GAACACTTCT TCATAGTTGA CCGCTTGAAG	1320
TCTTTAATTA AATACAAAGG ATATCAGGTG GCGGGGCTG AATTGGAATC GATATTGTTA	1380
CAACACCCCA ACATCTTCGA CGCGGGCGTG GCAGGTCTTC CCGACGATGA CGCCGGTGAA	1440
CTTCCCGCCG CCGTTGTTGT TTTGGAGCAC GGAAAGACGA TGACGGAAAA AGAGATCGTG	1500
GATTACGTCG CCAGTCAAGT AACAACCGCG AAAAAGTTGC GCGGAGGAGT TGTGTTTGTG	1560
GACGAAGTAC CGAAAGGTCT TACCGGAAAA CTCGACGCAA GAAAAATCAG AGAGATCCTC	1620
ATAAAGGCCA AGAAGGGCGG AAAGTCCAAA TTGTAAAATG TAACTGTATT CAGCGATGAC	1680
GAAATTCTTA GCTATTGTAA TCCTCCGAGG CCTCGAGGTC GA	1722

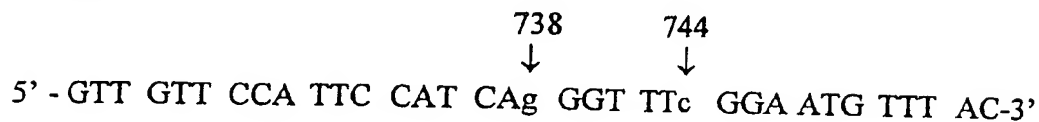
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Fig.7.**MUTAGENIC OLIGONUCLEOTIDES**Sequence ID No. 2

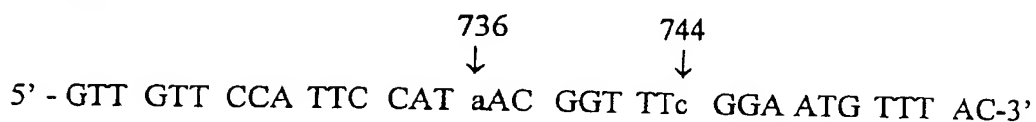
H245A:

Sequence ID No. 3

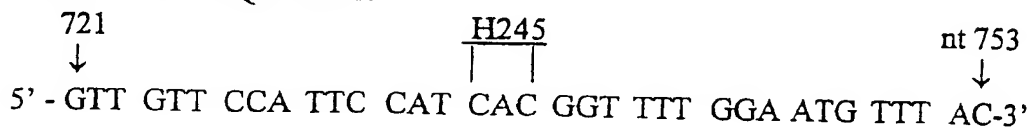
H245Q:

Sequence ID No. 4

H245N:

Sequence ID No. 5

WILD TYPE SEQUENCE:



nt numbering is from luc gene sequence, CAA
 |
 nt 1

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 550 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

Fig.8.

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Photinus pyralis*

(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 245

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

Met Glu Asp Ala Lys Asn Ile Lys Lys Gly Pro Ala Pro Phe Tyr Pro
1           5           10           15
Leu Glu Asp Gly Thr Ala Gly Glu Gln Leu His Lys Ala Met Lys Arg
20           25           30
Tyr Ala Leu Val Pro Gly Thr Ile Ala Phe Thr Asp Ala His Ile Glu
35           40           45
Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala
50           55           60
Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val
65           70           75
Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala Leu
85           90           95
Phe Ile Gly Val Ala Val Ala Pro Ala Asn Asp Ile Tyr Asn Glu Arg
100          105          110
Glu Leu Leu Asn Ser Met Asn Ile Ser Gln Pro Thr Val Val Phe Val
115          120          125
Ser Lys Lys Gly Leu Gln Lys Ile Leu Asn Val Gln Lys Lys Leu Pro
130          135          140
Ile Ile Gln Lys Ile Ile Ile Met Asp Ser Lys Thr Asp Tyr Gln Gly
145          150          155          160
Phe Gln Ser Met Tyr Thr Phe Val Thr Ser His Leu Pro Pro Gly Phe
165          170          175
Asn Glu Tyr Asp Phe Val Pro Glu Ser Phe Asp Arg Asp Lys Thr Ile
180          185          190
Ala Leu Ile Met Asn Ser Ser Gly Ser Thr Gly Leu Pro Lys Gly Val
195          200          205
Ala Leu Pro His Arg Thr Ala Cys Val Arg Phe Ser His Ala Arg Asp
210          215          220

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Fig.8 (Cont).

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Pro Ile Phe Gly Asn Gln Ile Ile Pro Asp Thr Ala Ile Leu Ser Val
 225 230 235 240

Val Pro Phe His Gln Gly Phe Gly Met Phe Thr Thr Leu Gly Tyr Leu
 245 250 255

- - - - - Met Thr Asp Phe Gln Gln Gln Leu

■ ⊙ ≡ L α L α ⊙ ⊙ ⊙ @
 Phe Leu Arg Ser Leu Gln Asp Tyr Lys Ile Gln Ser Ala Leu Leu Val
 275 280 285

Pro Thr Leu Phe Ser Phe Phe Ala Lys Ser Thr Leu Ile Asp Lys Tyr
 290 295 300